



Electron Uptake by Iron-Oxidizing Phototrophic Bacteria

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1 **Electron uptake by iron oxidizing phototrophic bacteria**

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16 **Summary:** The anoxygenic photoferroautotroph *Rhodopseudomonas palustris* TIE-1
17 accepts electrons from a poised electrode, which is uncoupled from photosynthesis, and
18 the PioABC system plays a role in electron uptake.

Abstract

Oxidation-reduction reactions underlie energy generation in nearly all life forms. While most organisms use soluble oxidants and reductants, some microbes can access solid-phase materials as electron-acceptors or -donors via extracellular electron transfer. Many studies have focused on the reduction of solid-phase oxidants. Far less is known about electron uptake via microbial extracellular electron transfer, and almost nothing is known about the associated mechanisms. Here we show that the iron-oxidizing photoautotroph *Rhodopseudomonas palustris* TIE-1 accepts electrons from a poised electrode, with carbon dioxide as the sole carbon source/electron acceptor. Both electron uptake and *ruBisCo* form I expression are stimulated by light. Electron uptake also occurs in the dark, uncoupled from photosynthesis. Notably, the *pioABC* operon, which encodes a protein system essential for photoautotrophic growth by ferrous iron oxidation, influences electron uptake. These data reveal a previously unknown metabolic versatility of photoferrotrophs to use extracellular electron transfer for electron uptake.

1 Introduction

2 Microbial metabolic activity substantially influences matter and energy flow
3 through the biosphere, and drives global biogeochemical cycles¹. Microorganisms have
4 broad metabolic capabilities, and can utilize chemically diverse, soluble substrates for
5 energy generation. Some microbes can also use solid-phase electron-acceptors and -
6 donors via a process called extracellular electron transfer (EET)²⁻⁵. Recent years have
7 been a watershed for microbial EET, with many studies focusing on the relevance of
8 EET in bioremediation and biotechnology^{6,7}. Although studies over the past few
9 decades have examined the role of microbial EET in donating electrons to metal oxides
10 and oxygen^{6,8-10}, the involvement of microbial EET in facilitating electron uptake has
11 come to fore only recently¹¹.

12 Studies show that mixed microbial communities facilitate cathodic reactions in
13 bioelectrochemical systems (BESs), implicating microbes in electron uptake¹¹. Recent
14 studies using pure cultures have shown that at least three microbes are capable of
15 taking up current from an electrode: *Sporomusa ovata*¹², *Mariprofundus ferrooxydans*
16 PV-1¹³, and *Shewanella oneidensis* MR-1¹⁴. Only the study performed on *Shewanella*
17 considered the genetic loci likely involved in electron uptake¹⁴. As such, the
18 mechanisms underlying electron uptake by microbes including *Shewanella* remain
19 poorly understood.

20 Characterizing how microbes take up electrons from solid-phase electron donors
21 is critical to our understanding of the ecological and evolutionary implications of this
22 process, as well as to any future biotechnology efforts such as electrosynthesis^{6,15}. The
23 establishment of genetic, genomic and metabolic studies in microbes that naturally take

up electrons via EET will lead to 1) identification of the associated genetic determinants, 2) the underlying molecular mechanisms, and 3) also facilitate experiments that examine the relationship between electron uptake and cellular metabolism.

Here we present data on our studies of *Rhodopseudomonas palustris* TIE-1 (TIE-1), a photoautotrophic microbe capable of accepting electrons from a variety of electron donors, including iron¹⁶⁻¹⁸ (Supplementary Fig. 1). We chose TIE-1 as the model organism because it uses ferrous iron, Fe(II), as an electron donor for photosynthesis (photoferrotrophy)¹⁶. Moreover, the metabolic versatility and genetic tractability of TIE-1 helps to readily interrogate the fundamental physiological relevance of electron uptake, including the degree and conditions under which TIE-1 takes up electrons, some genetic loci encoding systems involved in electron uptake, and the relationship between electron uptake and other physiological processes such as photosynthesis and carbon fixation. We observe that TIE-1 accepts electrons from a poised electrode, with carbon dioxide as the sole carbon source/electron acceptor. Both electron uptake and *ruBisCo* form I expression are stimulated by light. Electron uptake also occurs in the dark, uncoupled from photosynthesis. The *pioABC* operon, which encodes a protein system essential for photoautotrophic growth by ferrous iron oxidation, influences electron uptake.

Results

TIE-1 accepts electrons from a poised electrode

To characterize electron uptake by TIE-1, bioelectrochemical systems (BESs) were used. BESs are experimental systems where an electrode is submerged in a bioreactor, and is used to mimic the midpoint potential of solid-phase minerals^{3,6}. BESs provide an attractive alternative to using natural redox active minerals, allowing one to study microbial EET without confounding issues such as mineralogical changes during experimentation^{6,9,13,19,20}. The electrodes were poised at +100 mV vs. Standard Hydrogen Electrode (SHE) (Supplementary Fig. 2) as this potential is consistent with forms of Fe(II) utilized by TIE-1²¹.

TIE-1 was subjected to three treatments: 1) illuminated reactors with poised electrodes passing current (illuminated treatment); 2) non-illuminated reactors with poised electrodes passing current (dark treatment); and 3) illuminated reactors with electrodes at open circuit, passing no current (control treatment). The highest rates of current uptake by the TIE-1 wild-type (WT) were observed in illuminated treatments, up to $\sim 1.5 \mu\text{A cm}^{-2}$ (Fig. 1a). Cyclic voltammetry of the electrodes in the illuminated treatments revealed two modest but discernable cathodic peaks at +0.27 V and +0.4 V (vs. SHE) in the WT, which were absent in the abiotic control (Fig. 1b), suggesting the presence of redox active components in the illuminated reactors. Cathodic current was also observed in the dark treatments, suggesting that current uptake occurred under these conditions, though $\sim 70\%$ lower than when illuminated (Fig. 1a). We observed that cells attached to electrodes during all biotic treatments, with the highest viable cell densities occurring in the light treatment (Fig. 2a-b, Supplementary Fig. 3, Supplementary Table 1, Supplementary Table 2). Planktonic cells numbers increased during the course of the one-day incubations, though the increase in the WT illuminated

1 and control treatments were not significantly different at the end of these experiments
2 (Supplementary Table 3 and Supplementary Table 4).

3 To best capture the changes in gene expression during the onset of EET, these
4 treatments lasted ~24 hours to avoid issues that can arise during prolonged
5 experiments (e.g. differences in growth phase). Nevertheless, the apparent changes in
6 planktonic cell density would suggest that A) current was being used to support
7 planktonic growth; or B) an exogenous electron donor was available for growth.
8 Notably, in separate 5-day illuminated treatments, TIE-1 exhibited ten fold higher
9 densities than dark and control treatments. However, in these shorter-term illuminated
10 treatments, mass balance calculations suggest that the planktonic cell increase in the
11 bioreactors is two orders of magnitude lower than predicted if all current went to biomass
12 (Supplementary Note 1). Moreover, the trace concentrations of Iron present in the
13 medium (to support biosynthesis) could only account for up to 4.0×10^4 cells mL⁻¹ of the
14 observed cell increase (for calculations see Supplementary Note 2). Thus, there is an
15 electron sink other than biomass, and notably the gene expression data suggests that
16 this could be reductive CO₂ assimilation (discussed in detail below).

17 These data provide the first evidence of light-stimulated electron uptake by a
18 photoferrotroph, with some electron uptake also occurring in the dark, independent of
19 photosynthesis. Phototrophic microbes related to TIE-1 use photic energy for ATP
20 synthesis through cyclic electron flow, without the need for an electron donor²². An
21 electron donor is only required to produce reducing equivalents (NADPH) for cellular
22 metabolism most likely by reverse electron transfer²³. In the dark, no ATP can be
23 generated via photosynthesis but cellular metabolism continues²², thus requiring an

1 electron donor, which is likely represented by the observed dark current in our
2 experiments. The dark current also suggests that the electron uptake machinery is
3 independent (or can be uncoupled) of the cyclic photosynthetic apparatus. The increase
4 in electron uptake in the presence of light suggests that the ATP generated using the
5 energy of light is used by cellular processes, necessitating a higher level of electron
6 uptake.

8 **The *pioABC* operon plays a role in electron uptake**

9 Because these data reveal that TIE-1 accepts electrons from a solid-phase
10 conductor, we reasoned that it might employ conserved strategies to mediate this
11 electron uptake. Previous studies have shown that *pioABC* is essential for
12 photoferrotrophy, and have speculated that the Pio proteins might be involved in
13 electron transfer from Fe(II) to the electron transport chain^{17,21}. The *pioABC* operon
14 encodes the putative proteins PioA, a periplasmic decaheme cytochrome, PioB, an
15 outer membrane porin, and PioC, a periplasmic high potential iron-sulfur cluster protein
16 (HiPIP)^{17,21} (Supplementary Fig. 4). Using mutant studies and expression analysis we
17 directly tested whether the PioABC system plays a role in electron uptake. We observed
18 that $\Delta pioABC$ illuminated biofilms accepted 30% less current than the WT (Fig. 3a), and
19 the mutant illuminated biofilms were ~8 to 10 fold less dense than the WT
20 (Supplementary Table 2). Fewer $\Delta pioABC$ mutants colonized the electrode in the
21 illuminated treatments, which might result from an attachment defect. However, this was
22 not observed in the control treatments, i.e. in the absence of current, as the mutant cell

1 densities were comparable to the WT ($\Delta pioABC$ – 9.2×10^6 cells cm^{-2} , WT – 8.1×10^6
2 cells cm^{-2}).

3 If we assume that only attached cells contribute to electron uptake, then the
4 $\Delta pioABC$ mutants seem to accept more current per cell than the WT (Supplementary
5 Table 5 and Supplementary Table 6). This would imply that the $\Delta pioABC$ mutant cells
6 can take up electrons more actively, perhaps via compensatory changes. We posit,
7 however, that such an assumption is inaccurate as it disregards the potential
8 contribution of planktonic cells to electron uptake (Supplementary Fig. 2). Regardless,
9 these data collectively show that the Pio system influences electron uptake, though
10 other mechanisms of electron uptake clearly exist in TIE-1 as the mutant maintains
11 nearly 70% of the current uptake seen in the WT (Fig. 3a). Future studies should
12 examine the means by which the Pio system influences both phototrophic iron oxidation
13 and EET, and its potential role in governing attachment to poised electrodes.

14 We hypothesized that electron uptake might influence physiological systems that
15 play a role in EET as well as redox balance. Accordingly, we assessed the expression
16 of the target genes, including those encoding the PioABC proteins, across all
17 treatments. Expression of *pioA* in the WT illuminated biofilm was upregulated by ~48-
18 fold, while *pioB* and *pioC* showed more modest upregulation compared to the control
19 treatment (11- and 3-fold respectively; Fig. 3b). The observed levels of *pioA* in the WT
20 illuminated biofilm were well above those of the inoculum (grown on $\text{H}_2\text{:CO}_2$; Fig. 3b).
21 They were, however, comparable to gene expression observed during photoferrotrophic
22 growth on soluble Fe(II) in conventional culture apparatus. The decreased current
23 uptake of the $\Delta pioABC$ mutant, as well as the observed upregulation of the Pio genes in

1 the BES system, together suggest that the PioABC proteins may be involved in electron
2 uptake by TIE-1 under these conditions.

3 It should be noted that the PioABC module occurs in a number of anoxygenic
4 phototrophic microbes, which might show light-enhanced electron uptake as observed
5 in TIE-1 (Supplementary Table 7). Moreover, non-phototrophic ferrous iron-oxidizing
6 bacteria (FeOB) also possess the PioAB module, raising the question as to whether
7 these organisms perform light-independent electron uptake similar to the observed dark
8 current in TIE-1. The involvement of the MtrAB (related to PioAB¹⁷) system in the
9 electron acceptance by *Shewanella oneidensis* (MR-1) from a poised electrode also
10 suggests that this module might play a direct role in electron uptake¹⁴.

12 **Electron uptake stimulates expression of other genes**

13 We used expression analyses and microscopy to further examine TIE-1's
14 response to electron uptake. Exopolysaccharide (EPS) genes were highly upregulated
15 in the WT illuminated biofilms and, in some cases, in the $\Delta pioABC$ illuminated biofilms.
16 Expression analysis further showed that the *pioC* homolog (another HiPIP located
17 elsewhere on the chromosome) was upregulated (4-fold) in the WT illuminated
18 planktonic cells (cells not attached to the electrode present in the medium) compared to
19 the control treatment (Fig. 4 panel 1b) suggesting that the encoded protein might play a
20 role in planktonic cell increase under these conditions. Microscopy revealed that EPS
21 production was most abundant in illuminated biofilms (Fig. 4 panel 1a and 1a;
22 Supplementary Fig. 5a-b). Protein staining established the presence of extracellular
23 proteins in the cells attached to the electrodes (Supplementary Fig. 5c-d). Future

analysis on biofilm & planktonic cells, and the produced EPS will help determine the role of these elements in electron uptake.

***ruBisCo* form I expression increases during electron uptake**

Previous studies have shown that in organisms related to TIE-1, electron donors are required for generation of reducing equivalents, namely NAD(P)H, which serves as a reductant for cellular processes such as carbon fixation via the Calvin cycle^{22,24-26}. Ribulose-1, 5-bisphosphate carboxylase/oxygenase (RuBisCo), a key enzyme in the Calvin cycle, assimilates CO₂ into ribulose 1,5-bisphosphate yielding two molecules of 3-phosphoglycerate, which are eventually reduced by NAD(P)H to glyceraldehyde 3-phosphate in the reductive phase of the cycle²⁴⁻²⁶. TIE-1 and related microbes harbor genes encoding two forms of *ruBisCo*: form I and II²⁴⁻²⁶. We observed that *ruBisCo* form I was most highly expressed in WT illuminated biofilms (Fig. 4 panel Ic), and was typically higher than *ruBisCo* form II during conventional, photoautotrophic growth on hydrogen and Fe(II) (Fig. 4 panel Ic). Notably, *ruBisCo* form I expression was not induced in the dark treatment (Fig. 4, panel Ic). Previous studies on the regulation of *ruBisCo* form I expression in CGA009/10 (~99% identity to TIE-1¹⁶) shows that it is under exquisite control and is positively regulated by ATP and NAD(P)H, metabolites that indicate the energy status of the cell²⁷. During electron uptake in the presence of light, TIE-1 likely produces abundant ATP and NAD(P)H, which we posit leads to the increase in *ruBisCo* form I expression. In contrast, both these metabolites are likely lower in the dark, and thus *ruBisCo* form I expression decreases. It has also been suggested that RuBisCo form I can indirectly act as an electron sink (because increase

1 in its expression leads to higher production of 3-phosphoglycerate, which serves as the
2 substrate for the reductive part of the Calvin cycle) to maintain redox balance in
3 photosynthetic bacteria related to TIE-1^{25,26}.

4 5 **Discussion**

6 Our data provide a first glimpse on the ability of the photoautotrophic bacterium
7 *R. palustris* TIE-1 to accept electrons from a solid-phase electron donor. Because
8 photoautotrophs are exposed to diurnal cycles of light and dark conditions, we tested
9 the effect of illumination on the ability of TIE-1 to accept electrons. Our results show that
10 TIE-1 accepts electrons under both light and dark conditions, although light strongly
11 stimulates electron acceptance (Fig. 1a). The massive upregulation in genes that
12 encode for the *pioABC* system (encoding proteins that are suggested to play a critical
13 role in phototrophic iron oxidation by TIE-1¹⁷), as well as the decrease in current
14 observed in *pioABC* mutants, imply that the Pio proteins are engaged in electron
15 uptake. In contrast, the *pioABC* mutants appear to have an attachment defect to poised
16 electrodes thus exhibiting higher cell-specific electron uptake rates compared to the WT
17 (Fig. 3a, Supplementary Table 2 and Supplementary Table 6). This apparent pleiotropy
18 makes it difficult to ascribe a simple role to the *pioABC* system in electron uptake.

19 It should be noted that our experimental design does not allow us to exclude the
20 possibility that both the biofilm and the planktonic cells were engaged in electron
21 uptake, and the free living and biofilm lifestyles might be dynamic (Supplementary Fig.
22 2). The planktonic cells may have contributed to current uptake through direct
23 encounters with the electrode (these reactors were well stirred), or via soluble

1 compounds (we were unable, however, to detect any redox active compounds in the
2 spent medium; Supplementary Fig. 2 and Supplementary Fig. 6).

3 Transcriptomic analysis showed that *ruBisCo* form I expression was highest in
4 the poised illuminated electrodes (Fig. 4 panel Ic), suggesting that this enzyme could be
5 an indirect electron sink as has been observed in other related organisms^{25,26}. Because
6 RuBisCo is part of the Calvin Cycle, the carbon fixation pathway in TIE-1, it is plausible
7 that some of these electrons would go to biomass. Although we did see an increase in
8 the total cell density, there was no significant difference among these short-term
9 treatments, and the mass balance analyses suggest that biomass only accounts for a
10 modest amount of the total current passed (Supplementary Table 1, Supplementary
11 Table 2, Supplementary Table 3 and Supplementary Table 4). As mentioned, these
12 experiments were designed to be short in duration to avoid confounding factors
13 associated with growth and changes in growth phase. In light of TIE-1's typically modest
14 growth rates, it is likely that increases in biomass attributable to electron uptake during
15 these short-term treatments are below our limits of resolution.

16 In nature, electron uptake via EET could ameliorate metabolic dilemmas that
17 neutrophilic FeOBs, such as TIE-1, are known to face. FeOBs often contend with the
18 precipitation of insoluble iron oxides outside the cell, which are a byproduct of their
19 metabolic activity and potentially limit Fe(II) availability^{21,28}. TIE-1 produces poorly
20 crystalline Fe(III) hydroxides, which over time are abiotically transformed to the
21 (semi)conductive minerals goethite and magnetite^{16,29}. Conduction of electrons through
22 this matrix would allow TIE-1 (and potentially other FeOBs) access to electrons from
23 remote electron donors, including Fe(II) (Supplementary Fig. 7), via processes such as

electron conduction and iron atom exchange³⁰⁻³². Indeed, recent studies have shown that conductive minerals can facilitate electron transfer to microbes from remote electron donors (including other microbes)³³. These data extend this phenomenon to photoautotrophs, which is highly relevant because their restriction to the photic zone might hinder access to reductants in deeper, anaerobic layers^{22,34}. In addition to the ecological advantages of electron uptake via EET, there is substantial interest in exploiting photoautotrophs for both energy & biofuel generation¹¹, and identifying a genetically tractable photoautotroph that can use electric current as an electron donor holds promise in future electrosynthesis applications¹¹. While the ecological significance of EET is just coming to fore, our data illustrate the potential value of EET to microorganisms in nature, in particular photoautotrophs.

Methods

Bacterial strains, media, and growth conditions

Rhodospseudomonas palustris TIE-1 was grown as described previously¹⁸. For experiments, cells were pre-grown autotrophically on 80% hydrogen:20% carbon dioxide (H₂:CO₂) at 200 kPa, in fresh-water medium (FW) with 20 mM bicarbonate. The $\Delta pioABC$ strain used herein was constructed as previously described¹⁷. Phototrophic pre-growth was at 30°C using a 60W incandescent light source providing total irradiance of ~40 W m⁻². Bioelectrochemical reactor studies were conducted with FW medium (minimal salts medium lacking any added electron donors¹⁶⁻¹⁸) with 20 mM bicarbonate (sole carbon source¹⁶⁻¹⁸), buffered to pH 6.8 and with no exogenous electron-donor. All bacterial strains were routinely tested for purity by standard PCR

1 using primers indicated in Supplementary Table 8. Due to biological variation in the
2 cultivation effort, which resulted in different cell densities in the inoculum and prohibits
3 comparison across treatments, we ran a wild-type (WT) control in parallel with every
4 individual treatment to account for these differences. All comparisons between WT and
5 treatments are made using these paired runs.

6 7 **Bioelectrochemical system and conditions**

8 The bioelectrochemical systems (BES) consisted of new, acid-washed, combusted 350
9 mL borosilicate glass H-cell reactors equipped with two butyl rubber sampling ports in
10 the cathodic chamber (Adams and Chittenden Scientific Glass, Berkeley, CA, USA). A
11 vacuum clamp held the anodic and cathodic chambers together, and electrolytes were
12 separated using a cation-exchange membrane (Nafion[®] 117) with an active cross-
13 section of 20 cm² (Fuel Cell Store, Boulder, CO, USA). The working electrodes
14 consisted of spectroscopically pure 1/8" diameter graphite evaporation rods (SPI 01685-
15 FA, Structure Probe Inc, West Chester, PA, USA) that were mechanically polished with
16 1200 grit sandpaper, soaked in 5% HCl for 12 hours and stored in ultrapure deionized
17 water. The graphite rods were thoroughly dried prior to use by allowing the water to
18 evaporate. Each reactor was fitted with three graphite rods to provide a total immersed
19 projected electrode surface area of 18 cm². The rods were sealed with fittings and
20 ferrules on the reactor cap (Upchurch Scientific, Oak Harbor, WA, USA). Outside the
21 reactor, rods were electrically connected to one potentiostat using alligator clips
22 (described below). The counter electrode consisted of carbon cloth (Fuel Cell Store,

Boulder, CO, USA), which was mechanically attached to a titanium wire pierced through a rubber stopper (VWR) and suspended in the counter chamber.

Electrical conditions and cyclic voltammograms

The reactors were poised using custom-built potentiostats engineered for microbial chronoamperometry (Karma Electronics Inc., Somerville, MA, USA). Data was collected through a National Instruments DAQ (NI-6225) every 10 seconds using Labview SignalExpress software (National Instruments, Austin, TX, USA). Based on preliminary analyses of electroactivity in WT *R. palustris* TIE-1, the reactors were poised at +100 mV vs. Standard Hydrogen Electrode (SHE, -100 mV of the biological E_{pc} roughly at +200 mV vs. SHE) to assure cathodic conditions during the experiment. Importantly, this potential also ensures that a reductive Fe(III)/Fe(II) cycle is not established during these experiments (the redox potential at pH 7.0 of the Fe(III)/Fe(II) couple is +14 mV and electron transfer from an electrode poised at +100 mV will be an endergonic process)¹⁵.

Cyclic voltammetry (CV) was conducted using a Gamry R600 potentiostat (Gamry, Warminster, PA, USA). Biofilm CVs were obtained with a scan range of -100 mV to +900 mV vs. SHE at a rate of 20 mV per second. Supernatant voltammograms were obtained using a 3 mm diameter glassy carbon electrode (Part no. A-002012, BioLogic, Claix, France), under a N₂ atmosphere, scanned between 0 to +500 mV vs. SHE at 20 mV per second. We were unable to detect any electro-active soluble species in the 0.2 mM filtered spent medium (Supplementary Fig. 7). To assess the active surface area variability between electrodes, CVs were collected abiotically in fresh water medium. Potential is referenced to the SHE unless otherwise specified.

Description of bioelectrochemical set-ups

The distance between the working and counter electrodes was approximately 11 cm. Assembled BES reactors were sterilized by autoclaving in sterilization pouches and placed inside an anaerobic chamber (Coy, 2% hydrogen and palladium catalysts). Ag/AgCl reference electrodes were custom-made using glass tubing (4 mm KIMAX®), silver wire (0.5 mm diameter) and porous vycor tips (1/8" diameter, MF-2064, BASi). Reference electrodes were calibrated prior to each experiment, placed in the anaerobic chamber, sterilized with 70% ethanol, and placed in the counter chamber for the duration of the experiments. While inside the anaerobic chamber, media and counter buffer were added to the cathode and anode chambers, respectively. Inoculation of the BESs occurred inside the anaerobic chamber prior to transferring them outside the anaerobic chamber to establish electrical connections. The reactor system was purged continuously with a $1 \text{ cm}^3 \text{ min}^{-1}$ stream of $0.2 \text{ }\mu\text{m}$ filter-sterilized, deoxygenated gas stream of 80%:20% N_2 : CO_2 and 100% N_2 on the cathodic and anodic side, respectively, using a hypodermic needle immersed 1 cm below the media surface. The gases were deoxygenated using a high capacity oxygen trap lowering the oxygen levels to <0.01 ppm (Restek, Bellefonte, PA, USA). Each BES was individually housed with a fresh incandescent 60 W bulb providing a total irradiance of $\sim 40 \text{ W m}^{-2}$. Dark BESs lacked a bulb and were covered thoroughly with black paper to prevent light exposure. All working chambers were stirred gently with a magnetic bar and incubated at 30°C . All incubations, across all treatments, lasted 24 hours.

Sampling

The reactors were inoculated with 10 mL of cells in the mid-exponential phase of photoautotrophic growth on 80% H₂: 20% CO₂. One mL of media was withdrawn from the reactors immediately following inoculation and used for optical density (OD₆₆₀) determination with a 4802 spectrophotometer (Cole Parmer, Vernon Hills, IL, USA), and for pH measurements (Inlab[®] Expert Pro pH meter and probe, Mettler Toledo, Schwerzback, Switzerland). Four mL of culture was also withdrawn from the reactors for cell counts. Cells were fixed in 4% paraformaldehyde for cell counting (Electron Microscopy Sciences, Hatfield, PA, USA). At the end of each experiment, one of the electrodes was immediately dipped into RNA/*ater*[™] (Qiagen, Valencia, CA, USA) for RNA extraction. Also, 5 mL of planktonic cells were immediately preserved in RNA/*ater*[™] and filtered on a polyethersulfone (PES) membrane for RNA extraction (Corning, Tewksbury, MA, USA). All RNA samples were stored at -80°C. A second electrode was cut into ~5 mm pieces and transferred into fixatives or staining solutions for microscopic analyses (described below). Post experimentation, 1 mL of planktonic cells was sampled for OD₆₆₀ determination, and 2 to 4 mL for pH measurements. The remaining culture volume was then filtered on a 0.2 µm cellulose acetate filter (Corning, Tewksbury, MA, USA). After resuspension in 8 mL of media, these planktonic cells were pelleted in two 2 mL microcentrifuge tubes (18000 g for 10 min) and kept at -80°C along with the filtered spent medium.

Protein analysis

Subsamples for total protein analysis were processed in Prot loBind™ 1.5 mL or 2 mL microcentrifuge tubes (Eppendorf, Hauppauge, NY, USA). Trichloroacetic acid (TCA) precipitation was used as previously described¹⁸. The pellets were dried under vacuum for 1 hour to remove residual acetone, and then resuspended in 650 µL of 3 M Urea (ACS grade, Alfa-Aesar, Ward Hill, MA, USA). To ensure complete resuspension, the samples were incubated at 80°C for 3 days with frequent sonication in a sonic bath (FS30H, Thermo Fisher Scientific, Waltham, MA, USA). The Pierce BCA (bichinchoninic acid) Protein Assay Kit (Thermo Scientific, Rockford, IL, USA) was employed using the microtiter plate method for protein estimation as specified by the manufacturer with the provided bovine serum albumin as the standard protein. Each sample was quantified in triplicate. Absorbance at 562 nm was measured after 30 s shaking at 37°C using a Spectramax Plus 384 plate reader (Molecular Devices, Sunnyvale, CA, USA).

Fluorescence microscopy sample preparation and imaging

Sections of the electrode were placed into one of three solutions containing 1 µM 4',6-diamidino-2-phenylindole (DAPI, Life Technologies, Grand Island, NY, USA) as well as 1) LIVE/DEAD® stain (0.5 µM SYTO 9 and 3 µM propidium iodide, L7012, Life Technologies, Grand Island, NY, USA), 2) Exopolysaccharide (EPS) stain (200 mg L⁻¹ Concanavalin A and Alexa 488, Life Technologies, Grand Island, NY, USA), and 3) Protein stain (undiluted FilmTracer SYPRO Ruby Biofilm Matrix Stain, Life Technologies, Grand Island, NY, USA). Tubes were wrapped in aluminum foil and kept at room temperature for at least 30 minutes. Samples were then placed in 1X phosphate-buffered saline (PBS) in a glass-bottom dish, and imaged with a Zeiss 700

inverted confocal microscope with the following imaging lasers and Zeiss filters: 1) Live/Dead = 555 nm and 488 nm, SP490; 405 nm, SP555, 2) EPS = 488 nm and 405 nm, SP490 and LP490, 3) Protein = 555 nm, SP 490; 405 nm, SP555. This work was performed at the Harvard Center for Biological Imaging.

Scanning electron microscopy (SEM)

Sections of the electrode were cut using sterile techniques and immediately placed into a sterile microcentrifuge tube containing one of three solutions: 1) 5% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) in 1X PBS, 2) 2% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) in 1X PBS, and 3) 2% glutaraldehyde in 1X PBS with 0.15% Safranin O (Sigma-Aldrich, St. Louis, MO, USA), which has previously been shown to aid in EPS preservation⁷. Samples were held at 4°C for 24 hours before being subjected to ethanol dehydration by placing them in 35%, 50%, 70%, 95%, 100% ethanol (200 proof) in PBS or 0.1 M PBS solutions for ten minutes each. The 100% ethanol solution was changed five times, and the sample was left in ethanol for critical point drying (Autosamdri 815 A; Tousimis, Inc.) with a 15-minute purge time. The samples were adhered to SEM posts with carbon film tape and then imaged with a SEM at 5 kV (JEOL, Inc.). Cell counts for electrode samples were performed by analyzing microscopy fields taken at the same working distance (4.5 mm) to image, counting at least 500 cells or examining 12 fields of view if cell density was low and normalized to total area. This work was performed at the Harvard Center for Nanoscale Systems (CNS).

RNA isolation

For planktonic assessments, preserved cells were dislodged from the PES membrane before RNA extraction by vortexing for three minutes in a TRIS-EDTA (TE) buffer. For biofilm assessment, the cells were dislodged from the graphite by scraping with a sterile razor, then vortexing vigorously in TE buffer. RNA was extracted as described previously⁵. The RNA concentration was quantified using a NanoDrop ND1000 (Thermo Scientific, Wilmington, DE, USA).

RNA amplification

The RNA obtained from the biofilm on the graphite was cleaned with the MEGAclean™ Kit (Life Technologies, Grand Island, NY, USA) as per the manufacturer's guidelines. The purified RNA was precipitated using ammonium acetate. The reconstituted RNA was used as template for the MessageAmp™ II-Bacteria Kit as per the manufacturer's guidelines (Life Technologies, Grand Island, NY, USA).

Quantitative reverse transcription PCR (qRT-PCR)

Gene expression analysis was performed using qRT-PCR. The comparative Ct method was used as described previously to assess expression of the *pioABC* operon and other relevant genes⁵. Primer efficiencies were determined using the manufacturer's method (Applied Biosystems Inc. User Bulletin #2). *clpX* and *recA* were used as the two internal standards, which have been previously used and validated as internal standards¹⁸. The primers used for the assays are indicated in Suppl. Table S5. The iScript cDNA synthesis kit was used for reverse transcription (Biorad, Hercules, CA, USA). The iTaq

FAST SYBR Green Supermix with ROX (Biorad, Hercules, CA, USA) and the Stratagene Mx3005P QPCR System (Agilent, Santa Clara, CA, USA) were used for all quantitative assays.

Cell counting

The paraformaldehyde fixed samples were transferred into Amicon centrifuge filters (Amicon Ultrael 100k, regenerated cellulose membrane, Millipore, Carrigtwohill, CO, Ireland) and centrifuged for 10 min at 1000 g. The pellet was resuspended in PBS and washed twice. The cells were recovered by centrifugation of the Amicon in reverse position for 15 min at 3000 g. The resulting samples had less than 0.04% paraformaldehyde. Picogreen was added to the cells (Quant-iT PicoGreen[®] dsDNA, Life Technologies, Grand Island, NY, USA), and the cells were counted in 96 well plates along with 50 µL of Sphero™ AccuCount blank beads (Spherotek, Lake Forest, IL, USA). Cell-density was estimated with a LSRII flow cytometer (BD, Sparks, MD, USA) using a 488 nm laser. A calibration curve relating the ratio of cell events to beads events with cell-density was constructed by analyzing a dilution series of a cell sample, the density of which has been determined by microscopy (with a Helber Bacteria Cell counting chamber with Thoma ruling, Hawksley, Lancing, Sussex, UK).

ICP-MS

To measure the concentration of iron present in FW medium ICP-MS was performed using an Agilent 7700x ICP-MS with an octopole MS (Agilent, Santa Clara, CA, USA). Internal standards used were Germanium and Manganese, which were within

the detection limit of our system. The amount of iron in the basal medium was 4 μ M and ranged from 2-4 μ M in the spent medium.

***In silico* methods**

For identifying homologs of the PioABC proteins, delta-blast³⁶, FASTA³⁷ (<http://www.ebi.ac.uk/Tools/sss/fasta/>), and the IMG ortholog neighborhood search was used³⁸ (<http://img.jgi.doe.gov/cgi-bin/w/main.cgi>). Sequence similarity was calculated using EMBOSS matcher^{39,40} (http://www.ebi.ac.uk/Tools/psa/emboss_matcher/). The data reported is accurate as of October 2nd, 2012.

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- 2 EAP, EJG and PRG analyzed the data. EJG and CV contributed equally to this work.
- 3 AB and PRG wrote the manuscript with input from all co-authors.

4

1 **Conflict of interest:** The authors declare no conflict of interest.

2

Figure Legends:

Figure 1. Current uptake by wild-type *Rhodopseudomonas palustris* TIE-1

Panel a) Average current densities of *Rhodopseudomonas palustris* TIE-1 wild-type (WT) under illuminated and dark conditions. These values were obtained by averaging regions of >8 hours of stable current in each reactor and are reported as microamperes per centimeter squared ($\mu\text{A cm}^{-2}$). Error bars indicate standard deviations of these averages (n=3). Data reported are consistent with 10 independent runs. Panel b) Cyclic voltammograms of WT and ΔpioABC mutant after 96 hours of treatment in bioelectrochemical reactors with electrodes poised at +100 millivolts (mV) vs. Standard Hydrogen Electrode (SHE). Two sets of anodic-cathodic peak pairs were identified at 0.27 and 0.40 volts (V), respectively. The red trace depicts the difference in magnitude between the WT and the ΔpioABC mutant strain.

Figure 2. *Rhodopseudomonas palustris* TIE-1 cells attached to cathodes

Panel a) Fluorescence micrographs of a *Rhodopseudomonas palustris* TIE-1 wild-type (WT) illuminated biofilm (4',6-diamidino-2-phenylindole; DAPI stain). Scale bar = 10 μm . Panel b) Scanning electron micrograph (SEM) of a WT illuminated biofilm. Scale bar = 3 μm .

Figure 3. Current uptake and mRNA abundance of the *pioABC* operon by wild-type *Rhodopseudomonas palustris* TIE-1 and ΔpioABC mutant under various conditions

Panel a) Average current densities of *Rhodopseudomonas palustris* TIE-1 wild-type (WT) and ΔpioABC mutant under illuminated conditions. These values were obtained by averaging regions of > 8 hours of stable current in each reactor and are reported as

1 microamperes per centimeter squared ($\mu\text{A cm}^{-2}$). Error bars indicate standard deviations
2 of these averages ($n=3$). Data reported are consistent with 10 independent runs. Panel
3 **b)** mRNA abundance determined in the wild-type (WT) using qRT-PCR for the *pioABC*
4 genes. Cells were grown photoautotrophically with 5 mM FeCl_2 for photoferrotrophy.
5 Photoautotrophic growth on hydrogen as an electron donor was the inoculum. qRT-PCR
6 data are the averages \pm standard error for three biological replicates assayed in
7 triplicate. Illuminated = current with illumination. Control = no current with illumination.
8 Dark = current without illumination. Planktonic = cells not attached to the electrode
9 present in the medium. Biofilm = cells attached to the electrode.

10 **Figure 4. mRNA abundance determined in the *Rhodopseudomonas palustris* TIE-**
11 **1 wild-type (WT, panel I) and $\Delta pioABC$ (panel II) using qRT-PCR**

12 Panel **a)** Exopolysaccharide (*eps*) genes; panel **b)** *pioC* homolog; and panel **c)** *ruBisCo*
13 form I and II mRNA abundance. Cells were grown photoautotrophically with 5 mM FeCl_2
14 for photoferrotrophy. Photoautotrophic growth on hydrogen as an electron donor was
15 the inoculum. qRT-PCR data are the averages \pm standard error for three biological
16 replicates assayed in triplicate. Illuminated = current with illumination. Control = no
17 current with illumination. Dark = current without illumination. Planktonic = cells not
18 attached to the electrode present in the medium. Biofilm = cells attached to the
19 electrode. *eps I* = Rpal_3203, *eps II* = Rpal_3763, *eps IV* = Rpal_3771, *eps VI* =
20 Rpal_3777, *pioC* homolog = Rpal_4085.